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Of the various methods available for protein precipitation the classical ammonium sulphate has some disadvantages. The resulting protein solution often needs to be dialysed to obtain an ionic strength that allows ion exchange chromatography. This problem is avoided when using polyethylene glycol, PEG. Organic solvents, in particular ethanol and acetone, often produce extremely fine powder-like precipitates that are difficult to centrifuge and handle. They have also been shown often to cause partial denaturation of proteins, which can, for example, prevent subsequent crystallization. This is why organic solvents are not recommended as first-choice precipitating agents.

1.4.2.4 Liquid-Liquid Phase Extraction

A radically different way of making an initial fractionation is by partitioning in an aqueous polymer liquid-liquid two phase system[21]. These systems often contain polyethylene glycol, PEG, as one phase constituent and another polymer, such as dextran or even salt, as the other. Under favourable conditions it is possible to obtain the protein of interest in the upper, normally the PEG phase. The contaminating bulk protein as well as particles will become collected in the lower phase and can be removed by centrifugation. Particles sometimes stay at the interphase and are thus also removed in the centrifugation step. By covalent attachment of affinity ligands to PEG molecules these can be used for affinity partitioning. This technique is dealt with in detail in Chapter 11.

1.4.3 The Chromatographic Steps

1.4.3.1 Choice of Adsorbent

The first information of the chromatographic behaviour of a protein is often obtained most simply by preliminary analytical scale experiments, e.g., by gel filtration and by ion exchange chromatography using salt and pH gradients. In these runs approximate values of molecular size and ionic properties such as isoelectric points are obtained, information that is fundamental to the further planning of the work. A more thorough survey of the behaviour of the protein on various adsorbents can then be done using a panel of adsorbents. This can be carried out either in a panel of parallel columns or using tandem columns.

The parallel column approach has been developed by Scopes[22] for a panel of dye adsorbents. In this case he used up to 20 small columns containing various dye adsorbents. The columns were equilibrated with a predetermined application or starting buffer. A small volume of the protein extract was applied to each column and the protein content (280-absorption) and the activity in the effluent were measured. Then a predetermined terminating buffer was applied to each column and again the protein and enzyme activity in the effluent was determined. A column where the bulk of the proteins, but not the activity, was adsorbed was chosen as a "minus-column" while an adsorbent where the reverse happened was chosen as a "plus-column". These two columns in combination effected a considerable purification of the desired substance in the actual preparation. In a similar approach, a panel of parallel columns was used earlier by, e.g., Shaltiel[23] for evaluation of hydrophobic adsorbents. The technique can, however, be used for any set up of adsorbents such as different ion exchangers, the same ion exchanger under different conditions, thiol-gels, metal-chelating gels, etc. The elution of the columns can also be performed with more than two elution buffers. The purpose, however, is to get

a quick idea of the behaviour of a previously unknown protein and thus the set-up should not be enlarged beyond what can be handled easily in the laboratory.

If the adsorbents used have well defined and continuously increasing adsorption capacities for proteins in general the panel can also be arranged as tandem columns. This approach was used by Porath and co-workers for the immobilized metal ion (IMAC) adsorbents[24]. Here, three columns (Zn, Fe and Cu e.g.) were connected in series and a sample was pumped through all of them. After washing with starting buffer the three columns were disconnected and eluted separately, mostly using gradients. The approach requires that the first column adsorbs few of the proteins present whereas the last adsorbs almost all of them. This technique is not as generally applicable as the use of parallel columns.

1.4.3.2 The Order of the Chromatographic Steps

A priori one would expect that the order in which the different chromatographic steps are applied in a protein purification protocol is of minor importance. The total purification factor should be constant and the product of the factors obtained in each individual step should be independent of the other steps of the protocol. In the ideal case, where each chromatographic technique is utilized optimally with regard to the resolution and recovery, i.e., within the linear regions of the adsorption isotherms (see Chapter 2), with adequate sample volume to column volume ratios and with no adverse viscosity effects, this is probably true. However the real-life situations are always far from ideal or at least such that adaptation to ideality becomes highly impractical. For example, a fractionation gel filtration step can be optimized to give very high resolution (Chapter 3) but only at the cost of time and sample volume. To choose fractionation gel filtration as the first step when the sample volume might be much larger than the total volume of the column, means repetitive injections and excessive and impractical total process times, which would probably also be deleterious to the proteins in the sample solution. Likewise, to choose affinity chromatography on immobilized monoclonal antibodies as the first step would probably result in an extraordinarily high purification factor. However, the high cost of such adsorbents prohibits the use of large columns, which makes repeated injections of sample in smaller columns almost mandatory. This leads to long process times and the risk of product losses and/or modifications due to proteolytic attack. Proteolytic activity can also threaten the stability and life length of the actual immunosorbent. Furthermore, protein-based adsorbents are difficult to maintain to a sufficiently high degree of hygiene. There are limitations with regard to means for regeneration (washing) and sterilization (Chapter 10). This is why they should be saved for the later steps of the purification protocol.

The consequence of these considerations is that there are a number of practical rather than theoretical reasons why one should choose certain chromatographic techniques[26] for the early steps and others for the final steps of a protein purification process. The choice is primarily governed by the following parameters: (1) the sample volume, (2) the protein concentration and viscosity of the sample, (3) the degree of purity of the protein product, (4) the presence of nucleic acids, pyrogens and proteolytic enzymes in the sample and (5) the ease with which different types of adsorbents can be washed free from adsorbed contaminants and denatured protein. The last parameter governs the life length of the adsorbent and, together with its purchasing price, the material cost of the particular purification step.

In the light of what has been said above, the logical sequence of chromatographic steps would be to start with more "robust" techniques which combine a concentration

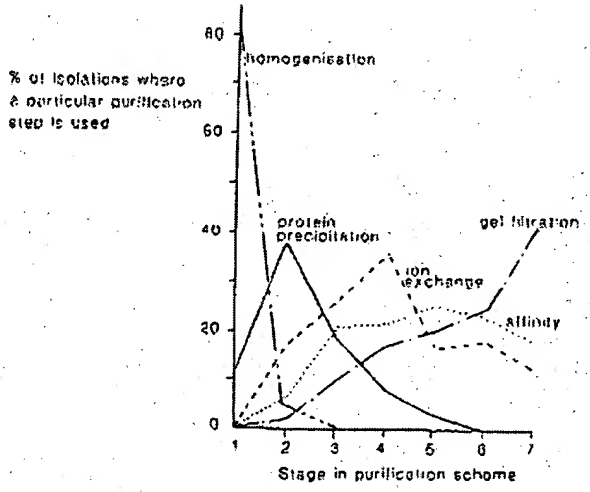

Figure 1-3. Analysis of the methods of purification used at successive steps in the purification schemes. The results are expressed as a percentage of the total number of steps at each stage. Adopted from ref. 26 by permission of the authors and publisher.

effect with high chemical and physical resistance and low material cost. The obvious candidates are ion exchange chromatography and to some extent hydrophobic interaction chromatography. As the latter often requires the addition of salt for adequate protein binding it is preferably applied after salt precipitation or after salt displacement from ion exchange chromatography thereby excluding the need for a desalting step. Thereafter, the protein fractions can preferably be applied to a more "specific" and more expensive adsorbent. The protocol is often finished with a gel filtration step (Fig. 1-3).

It is advisable to design the sequence of chromatographic steps in such a way that buffer changes and concentration steps are avoided. The peaks eluted from an ion exchanger can, regardless of the ionic strength, be applied to a gel filtration column. This step also functions as a desalting procedure which means that the buffer used for the gel filtration should be chosen so as to allow direct application of the eluted peaks to the next chromatographic step. The different chromatographies have, in practice, widely different capacities, even though it is possible to adapt several of the methods to a larger scale. However, in the initial stages of a purification scheme it is most convenient to start with the methods that allow the application of large volumes and which have the highest capacities. To this category belong, e.g., ion exchange chromatography and hydrophobic interaction, but any adsorption chromatographic method can be used to concentrate larger volumes, especially in batchwise operations.

1.4.4 The Final Step

The purpose of the final step is to remove possible aggregates or degradation products and to condition the purified protein for its use or storage. The procedure will thus be different depending on the fate of the protein. Aggregates and degradation products are preferably removed by gel filtration and if the protein is to be lyophilized